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Monoclonal antibodies to human A-I apolipoprotein and characterisation of cyanogen bromide fragments of APOA-I

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Several monoclonal antibodies to human A-I apolipoprotein were produced after immunising mice with pure delipidated apoA-I. These monoclonal antibodies were characterised for their ability to react with whole lipoproteins, apolipoproteins and fragments of apoA-I generated by cleavage with cyanogen bromide. The data suggest that production of monoclonal antibodies using apoA-I as antigen was influenced by two major epitopes subsequently localised to cyanogen bromide fragments 1 and 3, and have been designated antibodies $1 \rightarrow 5$ A-I^B and $6 \rightarrow 10$ A-I^B, respectively. Cyanogen bromide fragments were first purified to homogeneity before screening by competitive displacement or immunoblotting procedures. Definitive characterisation of one antibody series ($1 \rightarrow 5$ A-I^B) depended ultimately on Western blotting following isoelectric focusing of purified apoA-I fragments. This technique identified the epitope for these antibodies to fragment 1, an identification not fully concluded from competitive displacement studies. These studies have also revealed the presence of microheterogeneity in fragment 1 (as well as in fragment 4) of apoA-I, suggesting that structural variations in several regions may account for the polymorphism observed in this apolipoprotein.

Introduction

The development of monoclonal antibody technology has provided another useful tool for probing the structure and function of proteins of biological interest. Recently, several laboratories have reported on the production of monoclonal antibodies to different apolipoproteins, including apoB [1–3] apoA-I [2–6] apoA-II [7] and apoE [2,8]. These antibodies have had various applications, including immunoassays [2,8–13], and in investigations of receptor-binding domains of apolipoproteins [14,15], the heparin-binding sites of apoE [16] and the genetic polymorphism of apoB [17–19].

We have recently produced monoclonal antibodies from mice immunised with human apoA-I. Several of these have been shown to interact with different epitopes and some of these epitopes have been localised to particular fragments of A-I apolipoprotein resulting from cyanogen bromide cleavage. This paper describes the characterisation of these monoclonal antibodies and their use in identifying structural sites of this physiologically important apolipoprotein.

Materials and Methods

Experimental procedure.

HDL_2 (d 1.085–1.12 g/ml) and HDL_3 (d 1.12–1.21 g/ml) were obtained from human plasma (Red Cross blood bank) by ultracentrifugation as described previously [20]. After washing once at both lower and upper densities, or until free of apoE and albumin according to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunodiffusion analysis, the lipoprotein was dialysed and then delipidated with chloroform/methanol and ether [21]. HDL_3 apolipoprotein was then dissolved in 6 M urea in 0.05 M Tris HCl (pH 8.0), dialysed for 40 h against the same buffer, and applied to a Sephadex G-150 column (5 × 200 cm) previously equilibrated with the urea buffer. Fractions containing A-I and A-II apolipoproteins were separately pooled, dialysed and lyophilised and then analysed for purity by SDS-PAGE electrophoresis and immunodiffusion. If necessary

Abbreviations: SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; HRPO, horseradish peroxidase.

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apoA-I was rechromatographed until apparently homogeneous by SDS-PAGE and by immunochemical analysis. Amino acid composition and partial amino terminal sequence analysis confirmed the identity and purity of apoA-I (see below).

Immunisation of mice

Five female Balb/c mice were injected intradermally in multiple sites with 10–20 μg apoA-I in phosphate-buffered saline (PBS) emulsified with an equal volume of complete Freund's adjuvant (CSL, Melbourne). The mice were boosted at 2-weekly intervals until precipitation lines could be detected by immunodiffusion; generally 3–4 injections were required. 3 days before spleens were removed for cell fusion procedures, the animals were boosted intravenously with 10–20 μg (in 0.15 ml)of apoA-I in PBS (pH 7.4).

Monoclonal antibody production

The spleens were removed (one animal per fusion), the cells were extruded and, after washing, splenocytes were mixed with NS-1 mouse myeloma cells and fused, using the agent poly(ethylene glycol), according to the procedures described by Galfre and Milstein [22]. Following centrifugation and resuspension of cells in Dulbecco's modified Eagle's medium (DMEM) containing 15% foetal calf serum, the cell suspension was dispersed into 2 × 24 well plates and incubated for 24 h. The medium was changed to DMEM containing hypoxanthine, aminopterine and thymidine, and incubation continued for a further 14 days when medium without aminopterine was substituted. 14 days after fusing the supernatants were screened for the presence of apoA-I antibody by an enzyme-linked immunosorbent assay (ELISA). Cells from positive wells were cloned by the limiting dilution techniques in 96-well microtitre plates and positive clones were subcloned 2–3 times by limiting dilutions. Established cloned hybridomas (5×10^7) were injected into pristane-primed Balb/c mice. When animals showed evidence of ascitic fluid accumulation, they were sacrificed and the fluid collected.

Hybridoma screening

Supernatants were screened for the presence of antibody by the ELISA method as follows. Purified A-I apolipoprotein was diluted in coating buffer (0.05 M carbonate buffer, (pH 9.6) to 10 μg/ml and 50 μl aliquots were pipetted into wells of microtitre plates and incubated overnight at 37° C. After removal of the antigen, the wells were 'blocked' with 0.5% BSA in PBS. Test antisera or hybridoma supernatants were incubated in wells for 2 h at room temperature. Binding of antibody (from test samples) was detected using urease-conjugated rabbit anti-mouse IgG (Commonwealth Serum Laboratories) diluted 1 : 200, by incubation for 1 h at room temperature. After washing the plates, bound second antibody was detected by adding 50 μl urease substrate (CSL), to cause a colour change in the wells from yellow to purple.

Isolation of anti apoA-I immunoglobulin

Ascites fluid at 4° C was precipitated by the addition of ammonium sulphate to achieve a final 45% saturation. After dialysis, the protein concentration was estimated by the Lowry procedure [23]. In some cases, the partially purified immunoglobulin was further purified by chromatography through protein A Sepharose 4-B [24].

Immunoglobulin class determination

Monoclonal antibody immunoglobulin class was determined using a mouse monoclonal antibody isotyping kit (MISOTEST-CSL). Samples were added to wells coated with antibodies to specific heavy and light chains, and any positive reactions were detected using urease conjugated anti-mouse IgG Fab, and urease substrate as previously described above.

Radioiodination

10–20 μg of affinity-purified (protein A-Sepharose) monoclonal anti-apoA-I was labelled with ^{125}I using chloramine T, followed by reduction with sodium metabisulphite, as described previously [25]. After iodination the IgG was separated from unbound ^{125}I on a Sephadex G-50 column, eluted with 0.15 M NaCl in 0.02 M sodium phosphate, (pH 7.4) and dialysed against the same buffer.

Competitive displacement binding assays

96-well microtitre plates (Linbro) were coated with apoA-I, by incubation overnight with 50 μl per well of antigen (apoA-I, 10 μg/ml) in coating buffer ($NaHCO_3$, Na_2CO_3, (pH 9.6)). After removal of the antigen, the wells were washed twice with PBS, and then nonspecific binding sites were blocked with 100 μl 1% BSA in PBS containing 0.1% Tween 20 for 1 h at room temperature. The blocking buffer was removed, the wells washed a further three times with PBS/Tween 20. Serial 3-fold dilutions (from 1 mg/ml) (50 μl) of selected unlabelled monoclonal apoA-I antibody (ammonium sulphate fraction of ascites fluid) were added to some wells and 50 μl of ^{125}I-labelled monoclonal IgG (protein A purified, $(5–10) \cdot 10^4$ cpm, 10 ng protein) was added to each well and the plates incubated for 4 h at room temperature. The incubation medium was removed, the plates were washed four times with PBS/Tween 20 and well bottoms were sliced off for counting in a Packard auto γ-spectrometer. The results were expressed as $(B/B_0) \times 100$ where B = cpm bound minus nonspecific binding and $B_0 = {}^{125}I$ cpm bound in the absence of competing antibody minus nonspecific binding.

Cleavage of apolipoprotein A-I

Fragments of apoA-I were prepared by incubating 10 mg apoA-I with a 500-fold molar excess of cyanogen bromide [26].

Fragments were dissolved in 6 M urea and separated by gel filtration through Sephadex G-50 and eluted with 6 M urea in 0.02 M phosphate buffer (pH 2.3). Fractions were pooled, lyophilised and purified by reverse-phase HPLC as described elsewhere (unpublished data). The fragments were characterised by amino acid composition and sequencing as described below. Four pure CNBr fragments, labelled $CNBr_{1-4}$ were obtained and used in displacement experiments.

Characterisation of CNBr fragments by the competitive ELISA method

96-well flat bottomed plates (DYNATECH) were coated with A-I (250 ng/150 μl) in coating buffer, (pH 9.6), overnight at 37°C. After washing and blocking, 100 μl samples of competing protein (in duplicate) were added to wells in increasing dilutions across the plate followed by 50 μl monoclonal antibody (precipitated ascites, 100 ng/well). After incubating for 3 h at room temperature, the wells were washed and 150 μl of horseradish peroxidase (HRPO)-conjugated sheep anti-mouse immunoglobulin (diluted 1:2000) (Silenus) was incubated in each well for 1 h at room temperature. The amount of antibody bound was detected using horseradish peroxidase substrate mixture (0.2 mg/ml, *O*-phenylene diamine, 1.76 mM hydrogen peroxide) and absorbance measured at 492 nm on a Titertek Multiscan, MG.

Other procedures

SDS-polyacrylamide gel electrophoresis was performed on 10% slab gels (1.5 mm) as described by Weber and Osborn [27]. Western blotting was carried out after electrophoretic transfer onto nitrocellulose sheets according to the procedure of Burnette [28] using HRPO-conjugated second antibody obtained from Bio-Rad. Protein concentrations were estimated by the Lowry procedure [23]. Amino acid analysis was performed on the Beckman 6300 analyser following hydrolysis in 6 M HCl in sealed evacuated hydrolysis tubes for 22 h at 110°C. Sequencing of apoA-I was performed on the Applied Biosystems 470A sequencer with an on line 120 PTH analyser.

Isoelectric focusing was performing on 8 × 7 cm 7.5% polyacrylamide slab gels (0.75 mm) in the Mini Protean II electrophoresis chamber (Bio-Rad). Gel solutions contained 6 M deionised urea and 5% Pharmalytes (pH 4–6.5, Pharmacia) and proteins or peptides were dissolved in urea or pharmalytes (diluted 1:5) prior to focusing. At the completion of focusing (comprising prefocusing for 20 min at 100 V, loading of samples, then 30 min at 200 V and 2 h at 300 V) the gels were removed, and the proteins transferred to nitrocellulose (Bio-rad) by diffusion blotting at 37°C for 60 min. After blocking overnight at 4°C, the nitrocellulose sheets were incubated with monoclonal or polyclonal antibodies and bound antibody detected as described elsewhere [28].

Results

The results of three cell fusions produced dozens of clones that secreted anti-apoA-I antibodies. Systematic testing of these antibodies (described below) revealed two main epitopes which dominated the expression of monoclonal antibodies under our conditions. The results describe representative examples from many antibodies and large numbers were excluded from further characterisation since they did not recognise uniquely different antigenic sites of A-I apolipoprotein. All antibodies selected for further experiments were isotyped (see Materials and Methods) and found to be of the IgG$_1$ type with κ light chains. The monoclonal antibodies have been designated various numbers (e.g., 2 A-I^B etc.) where the prefix number refers to order of cloning, A-I (antibody to human apolipoprotein A-I) and B refers to Baker Medical Research Institute in order to distinguish the identity of these antibodies from similarly numbered antibodies produced in other laboratories.

Results of the competitive displacement experiments, carried out to compare antigenic determinants of the antibodies, are shown in Fig. 1. Since ammonium sulphate precipitated ascites was used as the source of unlabelled IgG fractions, the precise IgG concentration is unknown and the results are therefore expressed as the reciprocal of antibody dilution. When radioiodinated 7 A-I^B was incubated with increasing concentrations of unlabelled IgG fractions, some were found to compete for bound apoA-I. Only 9 A-I^B, and 7 A-I^B displaced the radiolabel and were similar in that displacement was achieved at highest concentrations of the antibody. Unlabelled 2 A-I^B and 3 A-I^B antibodies, however, produced no displacement, even at high concentrations. Since 2 A-I^B and 3 A-I^B are apparently unrelated to the epitopes recognised by 7 A-I^B, similar experiments were performed with radioiodinated 2 A-I^B (Fig. 1, middle panel). This was displaced by unlabelled 2 A-I^B and 3 A-I^B but not by 7 A-I^B and 9 A-I^b, which strengthened the observations of Fig. 1, top panel, that antibodies 7 and 9 A-I^B did not bind at or near the epitope for 2 A-I^B. Another antibody, 4 A-I^B was labelled and could be displaced with unlabelled 4 A-I^B, 2 A-I^B and 3 A-I^B but not by 7 A-I^B and 9 A-I^B (Fig. 1, bottom panel). These data (Fig. 1) suggest that antibodies 2, 3 and 4 A-I^B recognised one epitope, whereas 7 and 9 A-I^B share a different epitope of apoA-I.

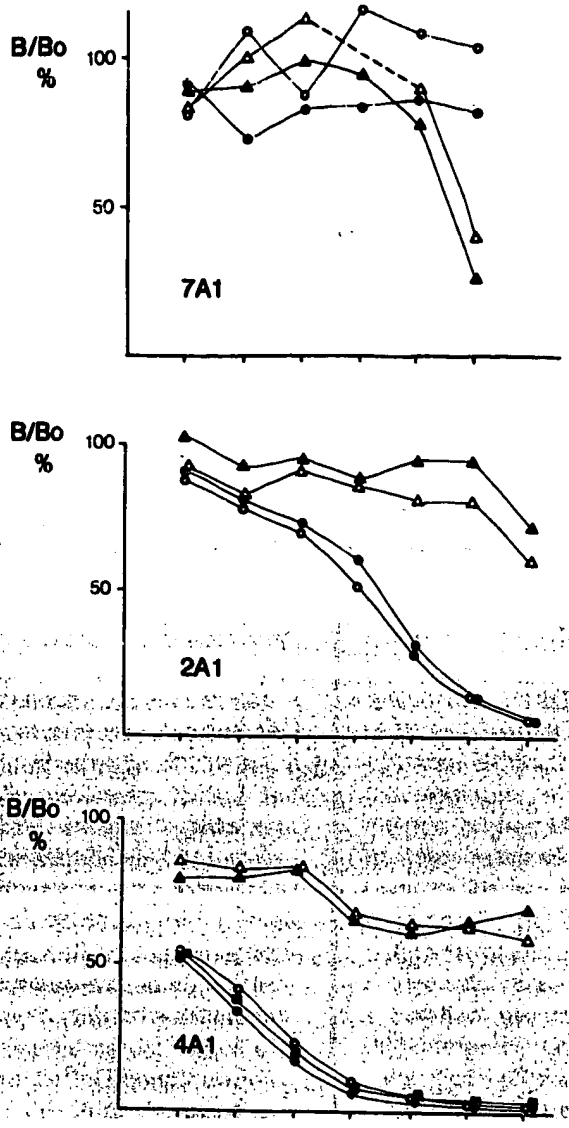

Fig. 1. Characterisation of monoclonal antibodies by competitive displacement. *Top Panel:* Antibody 7 A-I^B labelled with ^{125}I was incubated with unlabelled antibodies. ▲, 7 A-I^B; △ 9 A-I^B, ● 3 A-I^B, ○ 2 A-I^B and in multiwell plates coated with apoA-I. Bound radiolabel was detected as described. *Middle panel* ^{125}I-labelled antibody 2 A-I^B was incubated with unlabelled antibodies as described above. *Bottom panel* ^{125}I-labelled 4 A-I^B was incubated as described above, and in addition with unlabelled. ■, 4 A-I^B. The data is shown as % B/B_0 vs. reciprocal of antibody dilution. Starting concentrations of 7 A-I^B, 2 A-I^B and 4 A-I^B were 30, 100 and 1000 μg/ml, respectively.

Further competitive displacement experiments (not sh wn) revealed that antibodies 1 → 5 A-I^B shared one common epitope, and differed from 6 → 10 A-I^B which recognised another distinct site on apoA-I.

Location of epitopes

CNBr fragments of A-I apolipoprotein were obtained as described in Materials and Methods. The identity of four peptides generated by cyanogen bromide cleavage of apoA-I was established from their amino acid compositions which closely agreed with expected values. CNBr fragment 1, the amino terminal peptide, comprised residues 1–86; CNBr fragment 2 spans residues 87–112; CNBr fragment 3, residues 113–148; while CNBr fragment 4, the carboxy terminal peptide, spans residues 149–243.

The CNBr fragments were used in competitive displacement experiments in which the ability of intact apoA-I or fragments to compete with bound apoA-I for a monoclonal antibody was tested as described above. The binding of 2 A-I^B (Fig. 2) to apoA-I was displaced by whole apoA-I, a mixture of CNBr fragments (equivalent to A-I) and CNBr fragment 1 which competed very effectively at much lower concentrations than apoA-I or fragment 4. Fragments 2 and 3 did not compete for 2 A-I^B binding to solid-phase bound apoA-I. Binding of 3 A-I^B to apoA-I was also inhibited by apoA-I and by a mixture of fragments, whereas fragment 1 inhibited binding at much lower concentrations, suggesting it was a more effective competitor. CNBr fragments 2 and 3 did not compete and fragment 4 (not shown) produced only modest displacement at high concentrations.

Binding of 7 A-I^B to A-I apolipoprotein was inhibited by apoA-I, a mixture of CNBr fragments and fragment 3, whereas fragments 1, 2 and 4 were unable to compete. CNBr fragment 3 competed at lower concentrations than either apoA-I or a mixture of fragments. This would be expected as the competitors were used at equivalent protein concentrations and not at equimolar amounts.

9 A-I^B binding, in the same manner as 7 A-I^B, was inhibited by fragment 3, apoA-I and a mixture of all four fragments but not by fragments 1, 2 or 4.

These experiments suggested that the group of antibodies designated 6–10 A-I^B (represented by 7 and 9 A-I^B in these studies) recognised an epitope on apoA-I which could be localised to CNBr fragment 3. As noted above, McAbs 2 and 3 A-I^B recognised an epitope on CNBr fragment 1, but some competition for apoA-I by high concentrations of CNBr 4 was also observed. To resolve the uncertainty of which CNBr fragments are recognised by the antibody, individual CNBr fragments were subjected to isoelectric focusing and analysed by an immunoblotting procedure. The position of CNBr fragments 1, 3 and 4 as well as whole apoA-I were immunolocalised with anti-apoA-I polyclonal antibodies (Fig. 3, lanes 5–8) which also demonstrated that CNBr fragment 1 comprised several acidic is forms and fragment 4, at least 3 basic isoforms. A duplicate set of strips incubated with McAb 2 A-I^B only reacted with CNBr fragment 1 and not fragment 4 (lanes 2 and 4, respectively) establishing that the epitope recognised by 2 A-I^B resides in fragment 1.

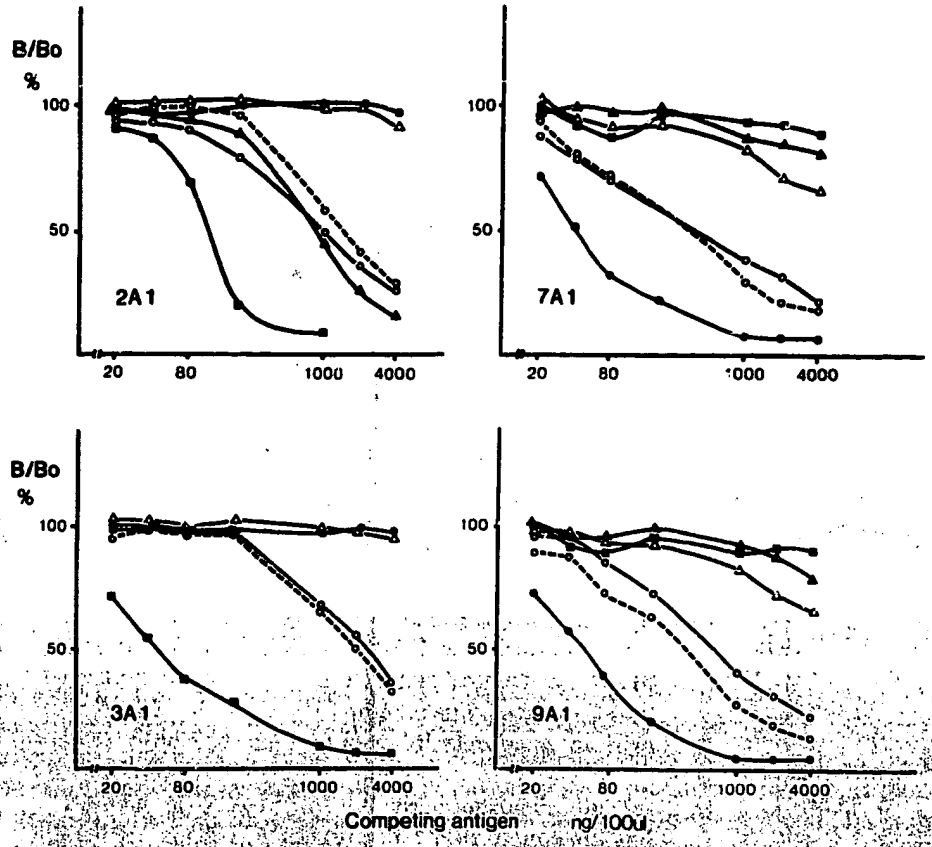

Fig. 2. Antigen binding sites on apoA-I for monoclonal antibodies. Antibodies were incubated (in multiwell plates, coated with apoA-I) with increasing concentrations of competing antigens ■ CNBr-1, △ CNBr-2, ● CNBr-3, ▲ CNBr-4, ○ apoA-I and ○——○, mixture of CNBr fragments. Antibody binding was determined by competitive ELISA as described in Materials and Methods.

Lipoprotein Specificity of McAbs

Competitive ELISA was used to determine the lipoprotein specificity of antibodies 2 A-I^B and 8 A-I^B (Fig. 4) as representative of the two groups which recognise the main epitopes described above. LDL and apoE did not compete with bound apoA-I, whereas apoA-I, HDL_2 and HDL_3 all competed for antibody binding to apoA-I.

HDL_2 and HDL_3 were used at similar protein concentrations based on the apoA-I content. Our results suggest that these epitopes on A-I in HDL_2 and HDL_3 are fully exposed as there was no difference in competition between HDL_2, HDL_3 and A-I apolipoprotein.

Apolipoprotein specificity

Monoclonal antibodies were also tested for monospecificity to apolipoproteins which are possible contaminants of the apoA-I used for immunising mice. Immunoblots of monoclonal antibodies 2 A-I^B, (a); 7 A-I^B, (b); and 9 A-I^B, (c), are shown in Fig. 5. After electrophoresis (in lanes 1–5) of a mixture of apolipoproteins (A-I, A-II, A-IV and E) and albumin, electroblotting and blocking, five strips were incubated with one of the McAbs and the position of the bound antibody detected with HRPO-conjugated second antibody. Strips 2–5 were then reincubated with polyclonal antibodies to apoA-II, apoE, apoA-IV and albumin, respectively, in order to locate positions of these apolipoproteins on nitrocellulose strips. The results show that each monoclonal antibody (strip 1) only reacted to apoA-I not against any other apolipoprotein or albumin present on the same strip.

Discussion

Of several dozen monoclonal antibodies produced against human apoA-I, only two distinct groups were identified by competitive inhibition studies. This suggested that production of these McAbs was influenced by two major antigenic determinants (epitopes) of apoA-I which were subsequently localised to CNBr fragments 1 and 3. In this respect, the present study shares the experience of other laboratories [4,29] which have also produced monoclonal antibodies reacting against amino-terminal or internal regions of apoA-I. Only two recent studies report on the production of McAbs which bind to the carboxy-terminus of apoA-I [6,30].

Furthermore, our experience demonstrates the importance of using purified CNBr fragments and analysing individual fragments using an appropriate sep-

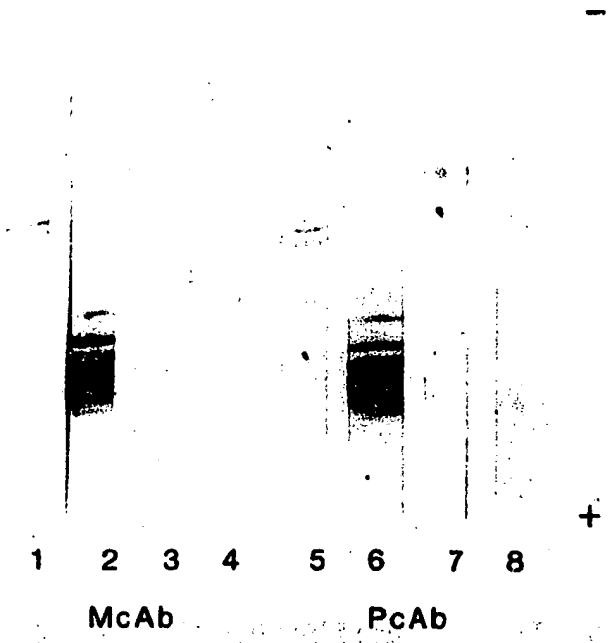

Fig. 3. Determination of antigen binding site by immunoblotting. Native apoA-I and CNBr fragments of apoA-I were separated by isoelectric focusing on urea-polyacrylamide gels (using pharmalytes in the range pH 4–6.5), electroblotted onto nitrocellulose strips and incubated with monoclonal antibodies as described in Materials and Methods. Lanes 1–4 and 5–6 were incubated with monoclonal antibody (McAb), 2 A-I^B and a polyclonal (PcAb) antibody, respectively. Lanes 1 and 4, apoA-I; lanes 2 and 6, CNBr fragment 1; lanes 3 and 7, CNBr fragment 3; lanes 4 and 8, CNBr fragment 4.

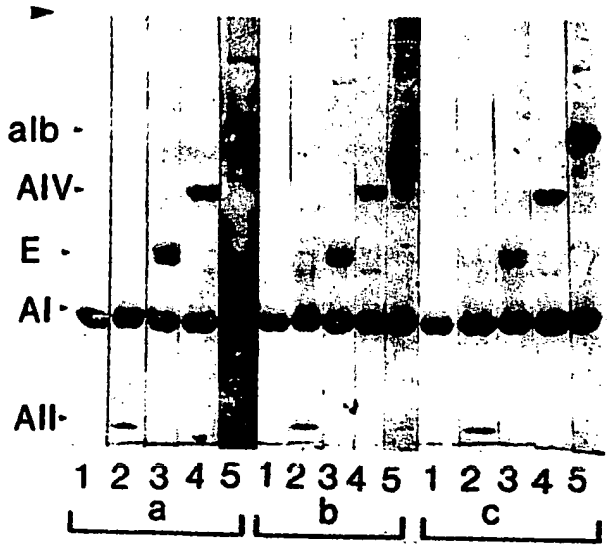

Fig. 5. Immunoblotting of apoA-I monoclonal antibodies. Mixtures of apolipoproteins A-I, E, A-II and A-IV and albumin were applied to SDS-polyacrylamide gels, transblotted onto nitrocellulose sheets and incubated with antibodies as described in Materials and Methods. All nitrocellulose strips were first incubated with an apoA-I monoclonal antibody followed by HRPO-conjugated second antibody and the colour developed. Separate strips were then reincubated with either polyclonal antibodies to (2) apoA-II, (3) apoE, (4) apoA-IV; or (5), albumin. (1) was not reincubated. Antibodies 1, 2 and 3 refer to 2 A-I^B, 7 A-I^B, and 9 A-I^D respectively.

aration system. Where the competitive inhibition studies failed to completely resolve identification of epitopes the isoelectric focusing system used here to separate CNBr fragments 1 from 4 confirmed that the epitope recognised by McAb 3 A-I^B resided on fragment 1 and not 4. The partial inhibition by fragment 4 could have been due to some contamination by fragment 1 or to the presence of a conformational epitope common to both CNBr fragments 1 and 4. The difficulty in producing McAbs reacting to the CNBr fragment 4 suggests that the strongest antigenic region of apoA-I resides in the amino-terminal segment, and that a higher proportion of antibodies reacting to these regions will dominate when the native polypeptide is used as antigen. This antigenic property of apoA-I may be masked when intact HDL is used to immunise animals, since Schonfeld et al. [31] reported that the C-terminal region of apoA-I was more reactive immunologically than the N-terminus probably reflecting the fact that this protein is more exposed on the surface of HDL.

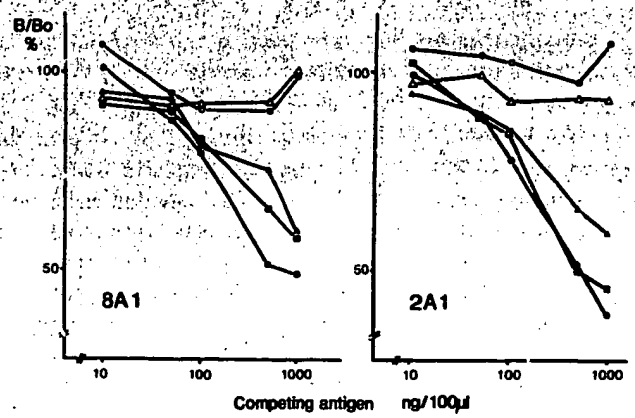

Fig. 4. Lipoprotein specificity of monoclonal antibodies in ELISA system. 8 A-I^B (left panel) and 2 A-I^B (right panel) were incubated with competing antigen △ apoE, ○ LDL, ▲ HDL_2, ● apoA-I and ■ HDL_3 in multiwell plates coated with apoA-I. Bound antibody was detected with HRPO-conjugated second antibody as described in Materials and Methods.

In the present study, fragments resulting from CNBr cleavage of apoA-I were first purified by reverse-phase HPLC before screening by the isoelectric focusing-Western blot system, thus confirming their identification as epitope markers. The technique enabled us to immunolocalise the epitope of McAb 3 A-I^B to CNBr fragment 1 and furthermore established the presence of microheterogeneity in fragment 1 as well as in fragment 4. This latter finding supports the suggestion of Weech et al. [4] that several regions of apoA-I are polymorphic and studies are presently in progress to further investigate the nature of this microheterogeneity.

Some of the physiological functions suggested for apoA-I include a role as activator of lecithin–cholesterol

acyltransferase [32], lipid binding [33] and serving as a ligand for a putative HDL receptor [34]. A global library of McAbs directed towards different epitopes of apoA-I will provide researchers with tools to probe the various active regions of this important plasma polypeptide and the antibodies reported in this study may, in conjunction with other panels of McAbs available elsewhere, serve a useful role in these investigations.

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